

EXHIBIT B

The Kinases MEKK2 and MEKK3 Regulate Transforming Growth Factor- β -Mediated Helper T Cell Differentiation

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SUMMARY

Mitogen-activated protein kinases (MAPKs) are key mediators of the T cell receptor (TCR) signals but their roles in T helper (Th) cell differentiation are unclear. Here we showed that the MAPK kinase kinases MEKK2 (encoded by *Map3k2*) and MEKK3 (encoded by *Map3k3*) negatively regulated transforming growth factor- β (TGF- β)-mediated Th cell differentiation. *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice showed an abnormal accumulation of regulatory T (Treg) and Th17 cells in the periphery, consistent with *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} naive CD4⁺ T cells' differentiation into Treg and Th17 cells with a higher frequency than wild-type (WT) cells after TGF- β stimulation in vitro. In addition, *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice developed more severe experimental autoimmune encephalomyelitis. *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells exhibited impaired phosphorylation of SMAD2 and SMAD3 proteins at their linker regions, which negatively regulated the TGF- β responses in T cells. Thus, the crosstalk between TCR-induced MAPK and the TGF- β signaling pathways is important in regulating Th cell differentiation.

INTRODUCTION

Naive CD4⁺ T cells must differentiate into distinct helper T (Th) cell lineages in order to mount appropriate immune responses against various classes of pathogens and to maintain tolerance to self-tissues. The differentiation of Th cell lineages, which include the Th1, Th2, Th17, and regulatory T (Treg) cell lineages, depends on signals derived from the T cell receptor (TCR) and the cytokine milieu of the local environment (Murphy and Reiner, 2002; Weaver et al., 2007). Dysregulation of Th cell differentiation has been associated with either immune deficiency or autoimmune disease (Bettelli et al., 2007). TGF- β is a pleiotropic cytokine crucial for many important biological processes including

adaptive immunity (Li and Flavell, 2008; Massagué, 1998). As a crucial cytokine for T cell-mediated immunity, TGF- β induces the differentiation and homeostasis of Foxp3⁺ Treg cells in the periphery (Chen et al., 2003), mediates the differentiation of Th17 cells in cooperation with IL-6 (Bettelli et al., 2006; Veldhoen et al., 2006), and suppresses the differentiation of Th1 and Th2 cell lineages (Gorham et al., 1998; Li and Flavell, 2008). Perturbation of TGF- β signaling specifically in T cells causes severe autoimmunity associated with excessive Th1 cell responses (Li et al., 2006; Marie et al., 2006).

TGF- β triggers a canonical signaling pathway through the TGF- β receptor-mediated phosphorylation of receptor regulated (R)-SMAD proteins SMAD2 and SMAD3 on conserved serine residues at their carboxyl (C) terminus (Derynck and Zhang, 2003; Massagué, 1998). Phosphorylated SMAD2 and SMAD3 subsequently bind to SMAD4 and translocate into the nucleus to mediate the transcription of target genes (Derynck and Zhang, 2003; Massagué, 1998). Many of the key components of this canonical pathway have been shown to be essential in mediating the TGF- β -dependent Treg and Th17 cell differentiation (Malhotra et al., 2010; Martinez et al., 2009, 2010; Tone et al., 2008). In addition to the C-terminal phosphorylation, R-SMADs can also be phosphorylated by mitogen-activated protein kinases (MAPKs) at a short region (termed linker region) that links the N-terminal MH1 domain (MAD homology domain) and the C-terminal MH2 domain, resulting in the suppression of the TGF- β signaling pathway (Derynck and Zhang, 2003; Kretschmar et al., 1999; Matsuura et al., 2005; Wrighton et al., 2009). The linker region phosphorylation of R-SMADs can also be mediated by other proline-directed kinases, such as CDKs and GSK3, resulting in either activation or suppression of R-SMAD activity (Alarcón et al., 2009; Guo et al., 2008; Millet et al., 2009; Wrighton et al., 2009). However, the regulation and significance of R-SMAD linker region phosphorylation in Th cell differentiation have not been studied.

MAPKs, which include the extracellular signal-regulated kinases (ERK1 and 2), the p38 (α , β , γ , and δ), the c-Jun N-terminal protein kinase (JNK1, 2, and 3), and the ERK5 MAPKs, are important intracellular signaling networks employed by all eukaryotic cells to transduce a wide spectrum of extracellular signals triggered by growth factors, cytokines, or stress factors (Dong et al., 2002; Su and Karin, 1996; Weston et al.,

2002). These MAPKs are activated by a three-kinase module that include a MAPK, a MAPK kinase (MAP2K), and MAP2K kinase (MAP3K) (Su and Karin, 1996). Previous studies have established the roles of individual MAPK pathways in T cell development, activation, and differentiation (Dong et al., 2002; Su et al., 1994), but the precise molecular mechanisms by which these MAPK modules function in Th cell differentiation remain unclear, nor is much known about their roles in either Treg or Th17 cell differentiation.

Among 19 MAP3Ks in the mammalian genome, MEKK2 and MEKK3 are two highly conserved members of the MEK kinase (MEKK) subgroup of the MAP3K superfamily. Transient expression of either *Map3k2* or *Map3k3* in vitro leads to their autoactivation and activates all four major MAPKs: ERK1 and ERK2, p38, JNK, and ERK5 (Cheng et al., 2000; Huang et al., 2004; Yang et al., 2001; Zhang et al., 2006). Previous biochemical and genetic studies show that MEKK2 and MEKK3 regulate either T cell activation or homeostasis, probably through a mechanism involving downstream MAPK activation (Guo et al., 2002; Wang et al., 2009). However, because both MEKK2 and MEKK3 are coexpressed in T cells as well as many other cell types and share substrate specificity (Cheng et al., 2000; Wang et al., 2009), they may have overlapping function(s) in T cells that cannot be revealed from studying single gene knockout mice.

Here we report that MEKK2 and MEKK3 negatively regulate TGF- β signaling in Th cell differentiation. Mice with MEKK2 and MEKK3 double deficiency in T cells had increased numbers of Treg and Th17 cells in the periphery. *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} (see below) naive T cells exhibited increased sensitivity to TGF- β stimulation in Treg, Th17, and Th1 cell differentiation because of a defect in ERK1 and ERK2 activation, resulting in hypophosphorylation of SMAD2 and SMAD3 at their linker regions. *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice developed more severe EAE disease and had more antigen-specific Th17 cells that were preferentially accumulated in the central nervous system. Together, our study uncovers a T cell-intrinsic regulatory mechanism for the TGF- β -mediated Th cell differentiation and demonstrates that T cell sensitivity to TGF- β is dynamically tuned by the TCR-induced activation of MEKK2 and MEKK3 through phosphorylation of SMAD2 and SMAD3 at their linker regions.

RESULTS

Generation of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} Mice

To study the roles of MEKK2 and MEKK3 in T cells, we generated MEKK2 and MEKK3 T cell double-deficient mice by crossing the *Lck-Cre;Map3k3*^{fl/fl} (referred as *Map3k3*^{Lck-Cre/-}) mice with the *Map3k2*^{-/-} mice (Guo et al., 2002; Wang et al., 2009). The resulting *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice have *Mekk3* deleted specifically in T cells and *Mekk2* deleted globally, resulting in the loss of both MEKK2 and MEKK3 only in the T cell lineage (Figure S1A available online).

Map3k2^{-/-}*Map3k3*^{Lck-Cre/-} mice were viable with no obvious abnormalities in nonimmune tissues (data not shown). The total number of thymocytes in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice was similar to that in wild-type (WT) mice, containing all the major subsets of thymocytes suggesting that MEKK2 and MEKK3 are not essential for the thymocyte development (data not shown).

Map3k2^{-/-}*Map3k3*^{Lck-Cre/-} mice had reduced numbers of CD4⁺ and CD8⁺ T cells in the periphery (data not shown), consistent with our recent finding that MEKK3 regulates T cell homeostasis in the periphery (Wang et al., 2009). Despite the reduction of total spleen cell number, the percentages of CD4⁺ and CD8⁺ T cells seemed only slightly reduced in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice (Figure 1A). However, when compared to WT mice there was a dramatic increase in the frequency of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} CD4⁺ T cells with an effector or memory-like (CD44^{hi}CD62L^{lo}) phenotype (Figure 1A).

Increased Numbers of Treg and Th17 Cells in the Periphery of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} Mice

Despite the increased effector or memory-like T cell population in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice, the percentage of Foxp3⁺ splenic CD4⁺ cells (Treg cells) was 2-fold higher than in the WT mice (Figure 1B). However, the absolute number of Foxp3⁺ cells in the periphery was not altered substantially (Figure S1B) because the total splenic CD4⁺ T cells in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice was only about half of that in the WT mice (data not shown). The frequency and number of Foxp3⁺ Treg cells in the thymus of the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice were not altered (data not shown), indicating that the increased frequency of Treg cells in the periphery of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice was not due to enhanced thymic production. Deletion of both MEKK2 and MEKK3 was required for the increased Treg cell percentage because neither *Map3k2*^{-/-} mice nor *Map3k3*^{Lck-Cre/-} mice manifested this defect (Figure 1C). Furthermore, the Treg cells in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice appeared functional and were able to suppress naive T cell proliferation in vitro with only a slightly reduced efficiency compared to their WT counterparts (Figure S1C).

We next examined the effector T cell subsets in the periphery of the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice. About 4%–5% of the peripheral *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} CD4⁺ T cells expressed IL-17A (Th17), whereas less than 0.5% of these cells were found in WT mice (Figure 1D). The total number of Th17 cells was also 3–4 times more in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice than in the WT mice (Figure S1B). Again, deletion of both MEKK2 and MEKK3 was required for the increased Th17 cells in the periphery (Figure 1E). The percentage of interferon- γ (IFN- γ)-producing CD4 T (Th1) cells was marginally increased but the total number of these cells was not increased in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice (Figure 1D and data not shown). The IL-4-producing T (Th2) cells were also not markedly changed in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice (data not shown). Similar data were obtained from analyzing the lymph node T cells (data not shown). Together, these results suggest that deletion of both *Map3k2* and *Map3k3* in T cells redirects Th cell differentiation favoring the Treg and Th17 cell lineages.

Because *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice have increased frequency of CD62L^{lo}CD44^{hi} T cells in the periphery, it is possible that the increased number of Th17 cells observed in these mice is simply a consequence of more effector cells in the periphery. To examine this possibility, we compared the frequencies of IL17A- and IFN- γ -producing T cells within the effector T cells (CD44^{hi}) between the WT and the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice. After normalizing to the CD44^{hi} population, the

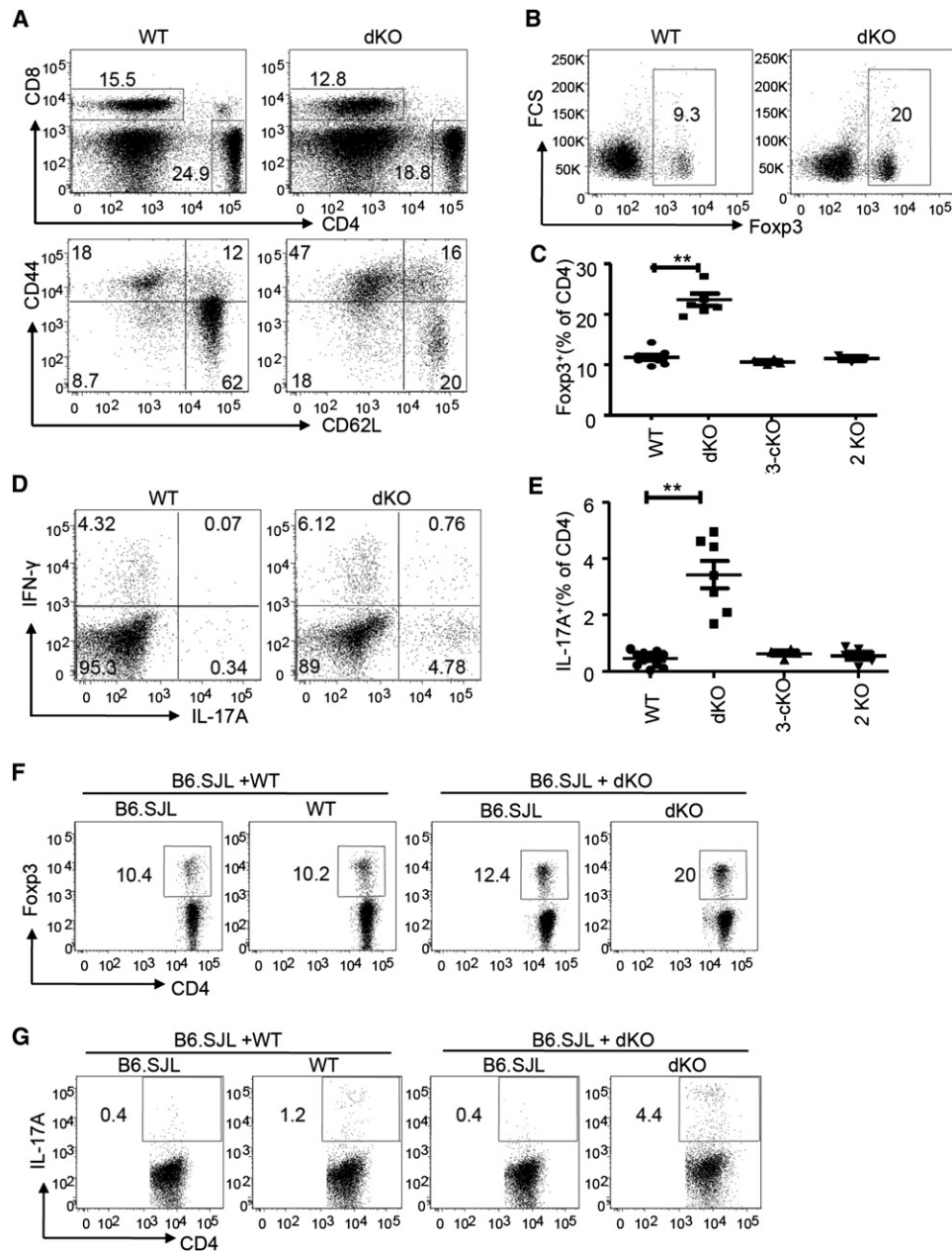


Figure 1. Loss of *Map3k2* and *Map3k3* in T Cells Results in Accumulation of Treg and Th17 Cells In Vivo

(A) Splenocytes from wild-type (WT) and *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} (dKO) mice were stained for CD4, CD8, CD44, and CD62L and analyzed by flow cytometry. The percentages of CD4⁺ and CD8⁺ T cells are shown at the upper panels. The gated CD4⁺ T cells were further analyzed for CD44⁺ and CD62L⁺ subpopulations (lower panels). Numbers in the profiles indicate the percentages of the gated populations.

(B) Splenocytes were stained for CD4 and Foxp3 to show the percentages (numbers in the gated boxes) of Foxp3⁺CD4⁺ T cells (Treg) in the spleen of WT and dKO mice. Data shown are from gated splenic CD4⁺ cells. FCS, forward scatter.

(C) Summary of splenic Foxp3⁺CD4⁺ T cells in WT (n = 8), dKO (n = 5), *Map3k3*^{Lck-Cre/-} (3-cKO) (n = 6), and *Map3k2*^{-/-} (2-KO) mice (n = 6). Error bars show the standard deviation. **p < 0.01 by two-tailed Student's t test.

(D) Ex vivo splenocytes were stimulated with PMA+ionomycin for 6 hr and then analyzed for IL-17A- and IFN- γ -expressing CD4⁺ T cells by flow cytometry. The data shown were gated on CD4⁺ splenocytes and the percentages of IL-17A- and IFN- γ -expressing CD4⁺ T cells from WT and dKO mice are shown in the quadrants.

(E) Summary of splenic Th17⁺ CD4⁺ T cells in WT (n = 10), dKO (n = 8), 3-cKO (n = 6), and 2-KO (n = 6) mice. Error bars show the standard deviation. **p < 0.01 by two-tailed Student's t test.

(F and G) *Rag1*^{-/-} mice were reconstituted with mixed bone marrow cells from B6.SJL (CD45.1⁺) and dKO (CD45.2⁺) mice, or from mixed B6.SJL (CD45.1⁺) and WT (CD45.2⁺) mice at roughly a 1 to 1 ratio. Total splenocytes in the recipient mice were analyzed 8 weeks later for CD4⁺Foxp3⁺ cells (F) or CD4⁺IL-17A⁺ cells (G) as indicated. Data shown are gated on CD4⁺CD45.1⁺ (B6.SJL) or CD4⁺CD45.2⁺ (WT or dKO) populations. Numbers next to the gated areas show the percentages of the gated population and the results are representative of six pairs of mice from two independent experiments.

Map3k2^{-/-}*Map3k3*^{Lck-Cre/-} CD4 T cells still had much higher frequency of Th17 cells than the WT CD4 T cells (8.76% versus 2.26%), whereas the frequency of IFN- γ ⁺ cells was indeed lower in the same subset of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} cells compared to that of the WT T cells (12.1% versus 21.9%) (Figure S1D). Thus, the increased frequency of Th17 cells in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice was probably not caused by the augmented numbers of CD62L^{lo}CD44^{hi} T cells in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice.

To further analyze the effect of MEKK2 and 3 deficiency on T cell differentiation in vivo, we directly compared the gene expression profiles of freshly isolated CD4⁺CD62L^{lo}CD44^{hi} cells between *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} and WT mice. By using an Illumina Microarray (Figure S1E, Table S1) and real-time quantitative RT-PCR (Figure S1E), we found that the expression of Th17 cell lineage-specific genes (*Il17A*, *Rorc*, and *Ccr6*) was markedly increased in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells, whereas the expression of Th1 cell lineage-specific genes (*Tbx21*, *Il12rb1*, *Ifng*, *Irf1*, *FasL*, etc.) was suppressed. These data together suggest that MEKK2 and 3 may inhibit Th17 cell differentiation while being required for optimal Th1 cell differentiation in the periphery.

MEKK2 and MEKK3 Suppress Treg and Th17 Cell Differentiation through a Cell-Intrinsic Mechanism

To test whether the defects in Treg and Th17 cell differentiation in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice were intrinsic to T cells, we reconstituted irradiated *Rag1*^{-/-} mice with *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} (CD45.2⁺) bone marrow cells mixed at a 1:1 ratio with WT congenic (CD45.1⁺) bone marrow cells. Eight weeks after the reconstitution, we analyzed the phenotypes of donor T cells in the periphery of the chimeric mice. The frequencies of the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} Foxp3⁺ CD4 cells (Figure 1F) and *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} IL-17A⁺ CD4 T cells (Figure 1G) in the spleens were consistently higher than their WT counterparts derived from the cotransferred congenic WT bone marrow cells in the same host, resembling the T cell phenotypes found in the parental *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice. We also generated bone marrow chimeric mice by transferring nonmixed control WT bone marrow cells or *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} bone marrow cells into the *Rag1*^{-/-} mice. Again, the peripheral *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells from those chimeric mice also fully recapitulated the phenotypes of T cells from the parental *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice, including the increased activated or memory-like T cells and increased frequencies of Treg and Th17 cells in the periphery (Figure S1F). These data together demonstrate that MEKK2 and 3 suppress Treg and Th17 cell lineage differentiation through a cell-intrinsic mechanism.

Map3k2^{-/-}*Map3k3*^{Lck-Cre/-} Naive T Cells Are Hypersensitive to TGF- β -Mediated Differentiation

To determine whether the Treg and Th17 cell phenotypes in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice were caused by defects in naive T cell differentiation, we examined in vitro differentiation of naive *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} CD4 T cells (CD4⁺CD62L^{hi}CD44^{lo}CD25⁻). Under the Treg cell differentiation condition, we found that at concentrations of 5 or 1 ng/ml of TGF- β , 82% or 60.8% of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} naive

T cells differentiated into the Foxp3⁺ Treg (iTreg) cells, respectively; whereas 33.2% or 14.2% of WT T cells differentiated into iTreg cells, respectively, under the same condition (Figure 2A). Interestingly, about 30% of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells spontaneously differentiated into iTreg cells without exogenous TGF- β whereas almost no WT iTreg cells were generated under the same condition (Figure 2A). To determine whether the spontaneous induction of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} iTreg cells was caused by the trace amount of TGF- β in the culture medium, a neutralizing antibody against TGF- β was added into the culture, which completely eliminated the iTreg cell differentiation from the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (Figure 2A).

To determine whether *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells were the source of TGF- β , WT and *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells were cocultured under Treg cell condition at 1:1 ratio without exogenous TGF- β . As shown in Figure 2B, only the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells but not the cocultured WT T cells acquired Foxp3 expression indicating that the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells did not overproduce TGF- β and the augmented *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cell differentiation into iTreg cells was not mediated by an autocrine and/or paracrine mechanism. Rather, the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells but not the WT T cells were able to respond to trace amount of TGF- β presented in the culture medium. Moreover, the in vitro generated *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} iTreg cells were as suppressive as the WT iTreg cells differentiated under the same condition (data not shown). Together, these data indicate that *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells are intrinsically hypersensitive to TGF- β stimulation as measured by iTreg cell differentiation.

Next, we examined the Th17 cell differentiation from the naive *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells in vitro with TGF- β and IL-6 (Bettelli et al., 2006; Veldhoen et al., 2006). We found that the Th17 cell differentiation from naive *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells was also augmented when compared to WT T cells (Figure 2C). Similar to the iTreg cell differentiation, differentiation of Th17 cells from naive *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells could be completely blocked by a TGF- β antibody. We found that substantial numbers of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (up to 9.7%), but not WT T cells, acquired Foxp3 expression by this Th17 cell differentiation procedure, with some cells coexpressing both Foxp3 and IL-17A (Figure 2C). Because strong TGF- β signal could induce Foxp3 expression under Th17 cell conditions (Zhou et al., 2008), this further suggests that the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells were much more sensitive to TGF- β stimulation than WT T cells. Under the Th17 cell differentiation procedure, the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells also expressed substantially higher amounts of ROR γ t than WT T cells (Figure S2A). Expression of other Th17 cell lineage-specific genes, such as *Il-17f*, *Il-23r*, and *Il-21*, but not *Il-22* or *Il-21r*, was also elevated in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (Figure S2B). Thus, MEKK2 and MEKK3 negatively regulate the TGF- β response in Th17 cell differentiation.

Because TGF- β signaling is also known to inhibit Th1 cell differentiation, the augmented TGF- β responsiveness in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells would predict an enhanced inhibition of Th1 cell differentiation by TGF- β . Indeed, by using the Th1 cell differentiation procedure, we observed a much more sensitive dose response of TGF- β inhibition of IFN γ -producing

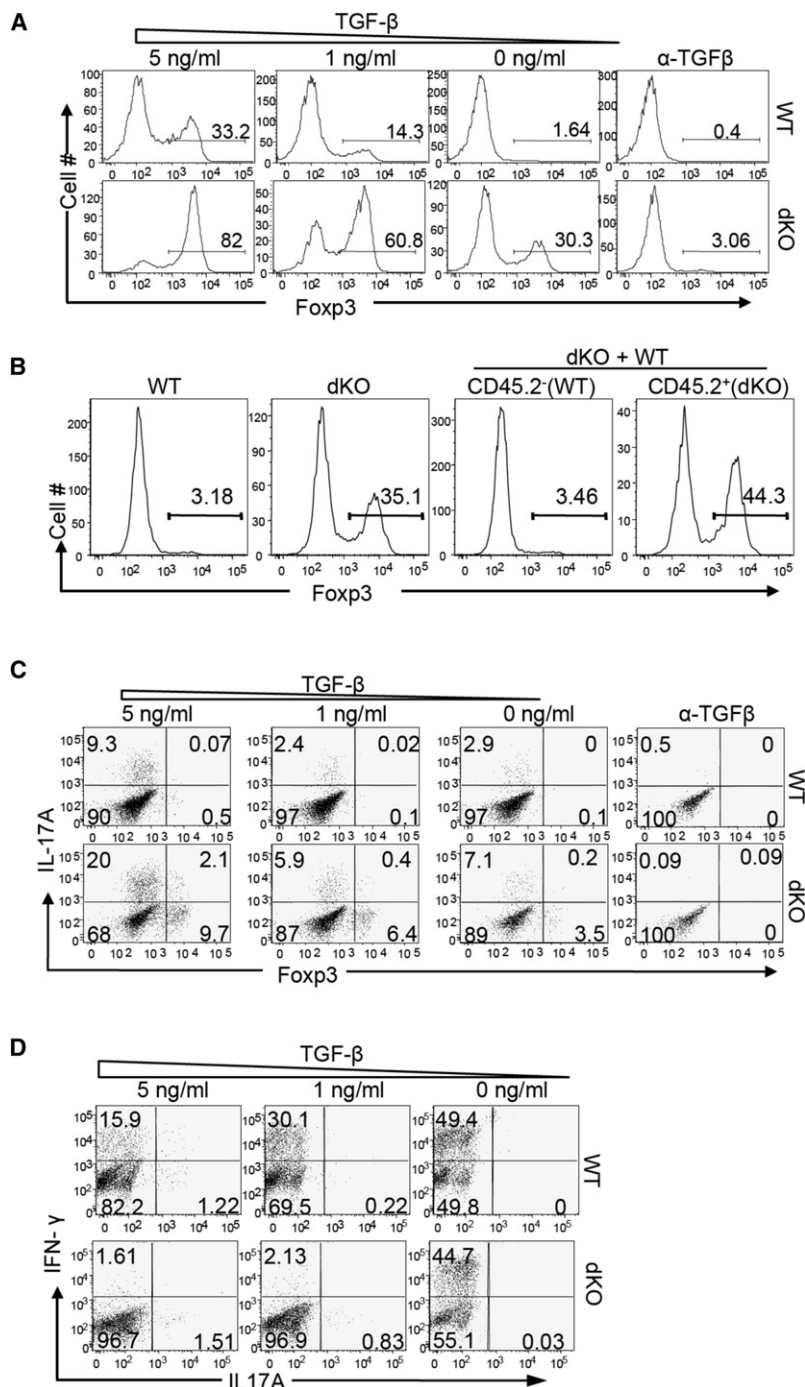


Figure 2. MEKK2 and MEKK3 Suppress the TGF- β -Mediated T Cell Differentiation In Vitro

(A) Naive CD4⁺ T cells (CD4⁺CD62L^{hi}CD44^{lo}CD25⁻) from WT and *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} (dKO) mice were differentiated into Fopx3⁺ Treg cells with the indicated concentrations of TGF- β . Induction of the Fopx3⁺ cells was analyzed 5 days after differentiation. When indicated, a TGF- β antibody (α -TGF β) (5 μ g/ml) was added throughout the culture.

(B) Naive dKO CD4⁺ T cells (CD45.2⁺) or WT B6.SJL CD4⁺ T cells (CD45.1⁺) were differentiated into Treg cells alone or in a mixed culture at a 1:1 ratio without exogenous TGF- β . The frequencies of differentiated Fopx3⁺ cells from dKO and B6.SJL CD4⁺ T cells were determined 5 days later.

(C) Naive CD4⁺ T cells from either WT or dKO mice were differentiated into Th17 cells with indicated concentrations of TGF- β and 20 ng/ml IL-6. At day 5, the differentiated cells were washed and restimulated with PMA+ionomycin for 4 hr, and the IL17A⁺ and Fopx3⁺ cells were determined by flow cytometry.

(D) Naive CD4⁺ T cells from either WT or dKO mice were differentiated into Th1 cells with 10 ng/ml IL-12 plus indicated concentrations of TGF- β . At day 5, the IFN- γ ⁺ and IL-17A⁺ cells were determined by flow cytometry as in (C). The numbers in the graphs indicate the percentages of the gated populations. Data are representative of either three (A, C, D) or two (B) independent experiments.

Taken together, these data demonstrate that the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} naive CD4⁺ T cells are hypersensitive to TGF- β -mediated induction of Treg and Th17 cell differentiation and to TGF- β -mediated inhibition of Th1 cell differentiation. Consistent with the in vivo data, the augmented TGF- β sensitivity in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells in vitro also required deletion of both *Map3k2* and *Map3k3* because neither *Map3k2*^{-/-} nor *Map3k3*^{Lck-Cre/-} naive T cells had augmented iTreg cell differentiation (Figure S2C). Moreover, CD4⁺CD25⁻ SP thymocytes from the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice were also hypersensitive to TGF- β stimulation during Th cell differentiation (data not shown). Additionally, we also examined Th2 cell differentiation and found that the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} CD4⁺ T cells appeared to have some reduction in the IL-4-induced Th2 cell differentiation (Figure S2D), which could be due to the increase of basal

TGF- β signals in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells that is known to inhibit Th2 cell differentiation (Gorelik et al., 2000).

Inhibition of TGF- β Signaling in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} Mice Blocks Treg and Th17 Differentiation In Vivo

The above results thus suggest that the increased Treg and Th17 cell differentiation in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice could be due to an augmented TGF- β signal in its T cells. To test this

Th1 cell differentiation from *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} cells (Figure 2D). Even a low concentration of TGF- β (e.g., 1 ng/ml) that had minimal effect on the WT T cells blocked most of Th1 cell differentiation of the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (Figure 2D). Furthermore, the induction of T-bet, a key transcription factor for Th1 cell lineage differentiation and a direct target of TGF- β (Gorelik et al., 2002), was inhibited more effectively by TGF- β in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells than in the WT T cells (data not shown).

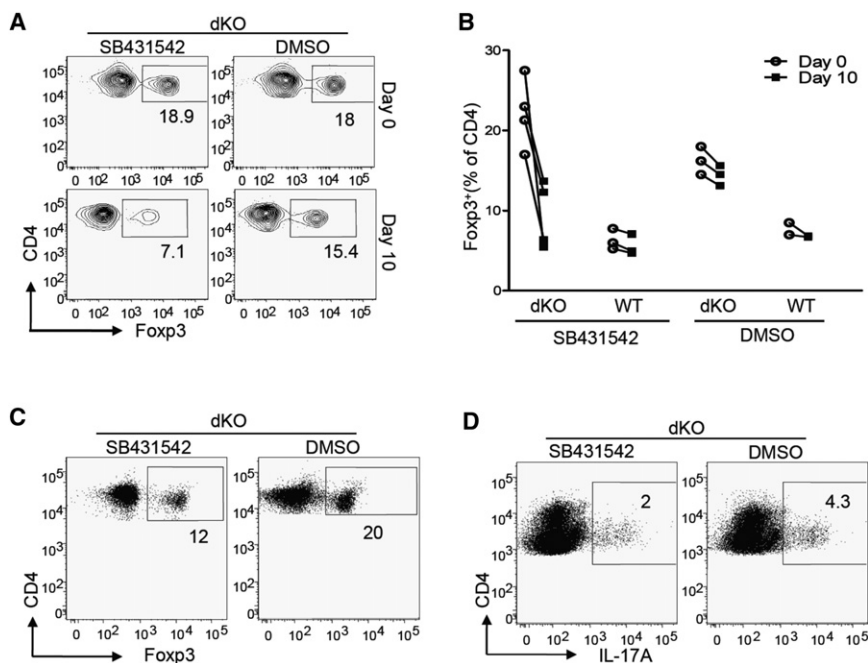


Figure 3. Inhibition of TGF- β Signaling in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} Mice Reduces Treg and Th17 Cells in the Periphery

(A) *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} (dKO) mice were treated with TGF β RI inhibitor SB431542 or DMSO every other day for 10 days. The Fopx3⁺ CD4⁺ T cells in the peripheral blood of dKO mice before the treatment (Day 0) and 10 days after the treatment (Day 10) were determined. The numbers next to the gated boxes show the percentages of the gated populations. Data are representative of three independent experiments. (B) Summary of Fopx3⁺ cells as the percentages of total CD4⁺ T cells in the peripheral blood of three pairs of wild-type (WT) and dKO mice before and after treatment with SB431542 or DMSO. Each symbol in the graph represents one individual mouse. (C and D) Splenic CD4 T cells from the dKO mice that were treated with SB431542 or DMSO were analyzed for the frequency of Fopx3⁺ population (C) or IL17A⁺ population (D) by flow cytometry. The numbers next to the gated boxes show the percentages of the gated populations. Data are representative of five mice per group from two independent experiments.

possibility, we treated the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice with a TGF- β receptor kinase inhibitor SB431542 (Inman et al., 2002). The inhibitor showed little effect on the ratios of naive versus effector or memory-like T cells in the periphery of either WT mice or the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice (data not shown). However, 10 days after the treatment, the frequencies of Fopx3⁺ Treg cells in the blood of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice were reduced to that in the WT mice (Figures 3A and 3B). Moreover, the percentages of Fopx3⁺ Treg cells and Th17 cells in the spleen of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice were also reduced after the inhibitor treatment (Figures 3C and 3D). As a control, the inhibitor had only marginal effect on the frequencies of Treg and Th17 cells in WT mice (Figure 3B and data not shown). These data suggest that the abnormal accumulation of Treg and Th17 cells in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice was due to the elevated TGF- β signals in T cells.

MEKK2 and MEKK3 Are Essential for the TCR-Induced Phosphorylation of R-SMAD at the Linker Region

Despite the antagonism between the Th17 and Treg cell lineage differentiation (Zhou et al., 2008), our in vivo and in vitro data indicated that the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells were prone to both lineage differentiations, suggesting that MEKK2 and 3 may inhibit the TGF- β pathway, which is shared for both Th17 and iTreg cell lineage differentiation. To confirm that the TGF- β signaling pathway in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells was indeed hyperactive, we examined the SMAD transcription activity in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells with CAGA₁₂-Luc, a synthetic SMAD-dependent luciferase reporter (Dennler et al., 1998). As expected, the induction of the reporter activity in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells was significantly higher than that in WT T cells (Figure 4A). However, the augmented SMAD reporter activity in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-}

T cells was not caused by the enhanced SMAD3 phosphorylation at the C-terminal-conserved sites at Ser423+425 (Figure 4B), because such phosphorylation was comparable between the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells and WT T cells (Figure 4C). Although TGF- β is known to induce a noncanonical pathway through MAPK activation (Derynck and Zhang, 2003), we did not observe substantial induction of ERK1 and 2 or p38 MAPK activation in response to TGF- β stimulation in either WT or *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (data not shown).

In addition to the C-terminal phosphorylation, R-SMADs can also be phosphorylated by MAPKs at the linker region (Figure 4B). We thus compared the phosphorylation of SMAD2 and SMAD3 at their linker regions between *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells and their WT counterparts. T cell activation by anti-CD3 and anti-CD28 or with PMA and ionomycin treatment led to rapid and strong phosphorylation of SMAD2 or SMAD3 at the linker regions in the WT T cells (Figures 4D and 4E). Such phosphorylation was severely impaired in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells, indicating that MEKK2 and MEKK3 are crucial for the TCR-induced phosphorylation of SMAD2 and 3 at their linker regions.

To confirm that MEKK2 or MEKK3 were capable of inducing R-SMAD linker region phosphorylation, and to verify that their activation could suppress R-SMAD transcriptional activity, we coexpressed *Map3k2* or *Map3k3* with SMAD3 in 293T cells. Transient expression of either MEKK2 or MEKK3 was sufficient to induce SMAD3 phosphorylation (Figure 4F) and SMAD2 (data not shown) at the linker regions. Furthermore, expression of either *Map3k2* or *Map3k3* was also able to inhibit the TGF- β -induced CAGA₁₂-Luc reporter activity (Figure 4G). Moreover, the transcriptional activity of a mutant SMAD3, EPSM-SMAD3, containing Ser or Thr to Ala or Val mutations in its linker region (Calonge and Massagué, 1999) was more resistant than the

Immunity

MAPK Limits TGF- β Responses in Developing Th Cells

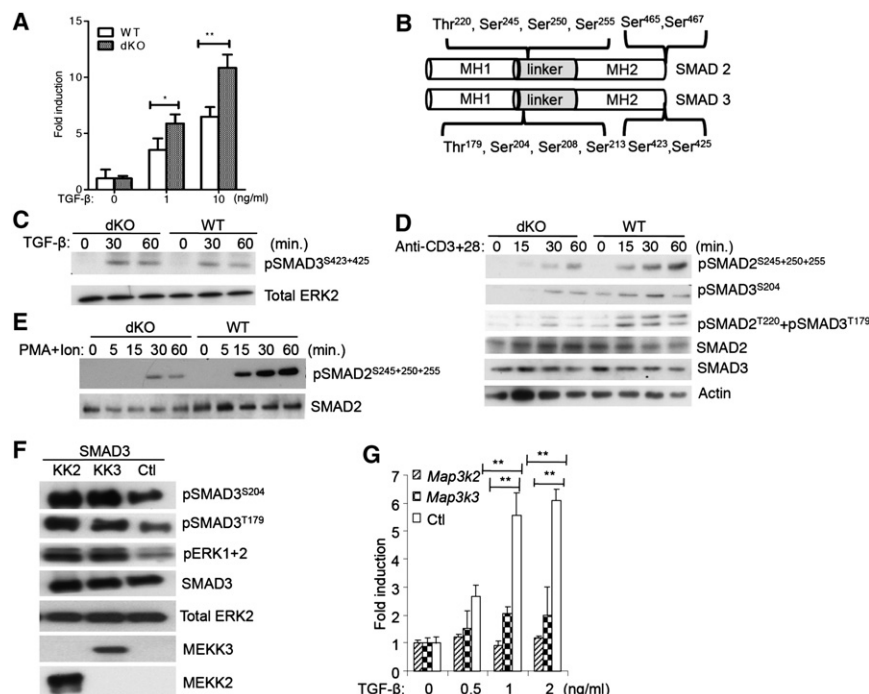


Figure 4. MEKK2 and MEKK3 Mediate R-SMAD Linker Phosphorylation and Inhibit SMAD Transcriptional Activity

(A) CAGA₁₂-Luc plasmid was nucleofected into WT or dKO CD4⁺ T cells. Four hours after nucleofection, cells were stimulated with CD3+CD28 antibodies with or without TGF- β as indicated. Luciferase activity was determined 16 hr later and normalized to the activity of non-TGF- β -stimulated cells.

(B) An illustration of known phosphorylation sites in SMAD2 and SMAD3. MH1 and MH2, MAD homology domain 1 and 2; linker, the sequence that links MH1 and MH2 domains.

(C) WT or dKO CD4⁺ T cells were stimulated with 10 ng/ml TGF- β as indicated and the SMAD3 phosphorylation at the C terminus (Ser423/425) was determined by immunoblotting. The ERK2 level is used as a loading control.

(D) WT or dKO CD4⁺ T cells were stimulated with CD3+CD28 antibodies (Anti-CD3+28) for the indicated time periods. Phosphorylation of the SMAD2 and SMAD3 linker regions at SMAD2 Ser245+250+255, SMAD3 Ser204, SMAD2 Thr220, and SMAD3 Thr179 was determined by immunoblotting. Total SMAD2 and SMAD3 protein levels were determined by immunoblotting. Actin level is shown as a loading control.

(E) WT and dKO CD4⁺ T cells were stimulated with PMA+ionomycin (PMA+ion) for the indicated time periods. Phosphorylation of the SMAD2 linker region and total SMAD2 protein was determined by immunoblotting.

(F) SMAD3 expression vector was cotransfected with either empty expression vector (Ctl) or expression vectors for *Map3k2* (KK2) or *Map3k3* (KK3) into 293T cells. Twenty-four hours after transfection, phosphorylation of SMAD3 at the linker region was determined. ERK1 and 2 activation and expression of SMAD3, MEKK2, and MEKK3 in the same transfection was determined by immunoblotting as indicated.

(G) CAGA₁₂-Luc reporter plasmid was cotransfected into 293T cells with either empty expression vector (Ctl) or expression vectors for *Map3k2* or *Map3k3*. Twenty-four hours after transfection, cells were stimulated with the indicated concentrations of TGF- β for additional 12 hr. The reporter activity without TGF- β stimulation was arbitrarily set as value 1 and used for normalization of the relative reporter activity. Data shown are representative of three independent experiments. Error bars in (A) and (G) show standard deviation. **p < 0.01 by two-tailed Student's t test.

WT SMAD3 to the MEKK2- or MEKK3-mediated suppression (Figure S3), further indicating that MEKK2 and 3 suppress the R-SMAD activity at least in part through the linker region phosphorylation.

ERK1 and ERK2 Mediate MEKK2- and MEKK3-Dependent Phosphorylation of R-SMAD at Their Linker Regions

Because MAPKs are known to phosphorylate R-SMAD at the linker regions, it is possible that MEKK2 and 3 deficiency may result in defective MAPK activation, which in turn leads to the impaired R-SMAD phosphorylation. Indeed, we found that activation of ERK1 and 2 and p38 MAPKs was severely impaired in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells as compared to that in WT T cells after TCR stimulation (Figures 5A and 5B). Consistent with the reduced activation of ERK1 and 2, induction of ERK1 and 2 target genes after TCR stimulation, such as *Egr1* and *c-Fos*, was greatly diminished in naive *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells than that in WT T cells (Figure S4A). The JNK was marginally activated and appeared not affected in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (data not shown).

Because both the ERK1 and 2 and p38 activation was impaired in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells, we then determined whether both of them or either one of them might

mediate the TCR-induced phosphorylation of R-SMAD at the linker region. Inhibition of ERK1 and 2 but not p38 MAPK in WT T cells greatly reduced the TCR-induced or PMA-induced phosphorylation of SMAD2 and SMAD3 at their linker regions (Figures 5C and 5D). Inhibition of both ERK1 and 2 and p38 did not further reduce the SMAD linker region phosphorylation compared to inhibition of ERK1 and 2, suggesting that there was no synergistic effect between ERK1 and 2 and p38. Because other kinases such as GSK3 and the CDKs are also able to phosphorylate SMAD2 and 3 linker region under certain conditions (Wrighton et al., 2009), to rule out their possible involvement in this process, we treated WT T cells with either GSK3 inhibitor (SB-216763) or CDK inhibitor (flavopiridol) and found that neither of them was able to block the anti-CD3-induced SMAD linker region phosphorylation (Figure S4B). Finally, although some cytokines such as IL-2 were able to induce ERK1 and 2 activation in T cells, IL-2 did not induce R-SMAD linker region phosphorylation (Figure S4C). Thus, ERK1 and 2 are responsible for the TCR-induced, MEKK2- and 3-dependent phosphorylation of SMAD2 and 3 at the linker region.

To further examine the physiological significance of R-SMAD linker region phosphorylation in T cells, we blocked the R-SMAD linker region phosphorylation via inhibition of ERK1 and 2 and determined its effect on Treg cell differentiation. As shown in Figure 5E, inhibition of ERK1 and 2 activation markedly

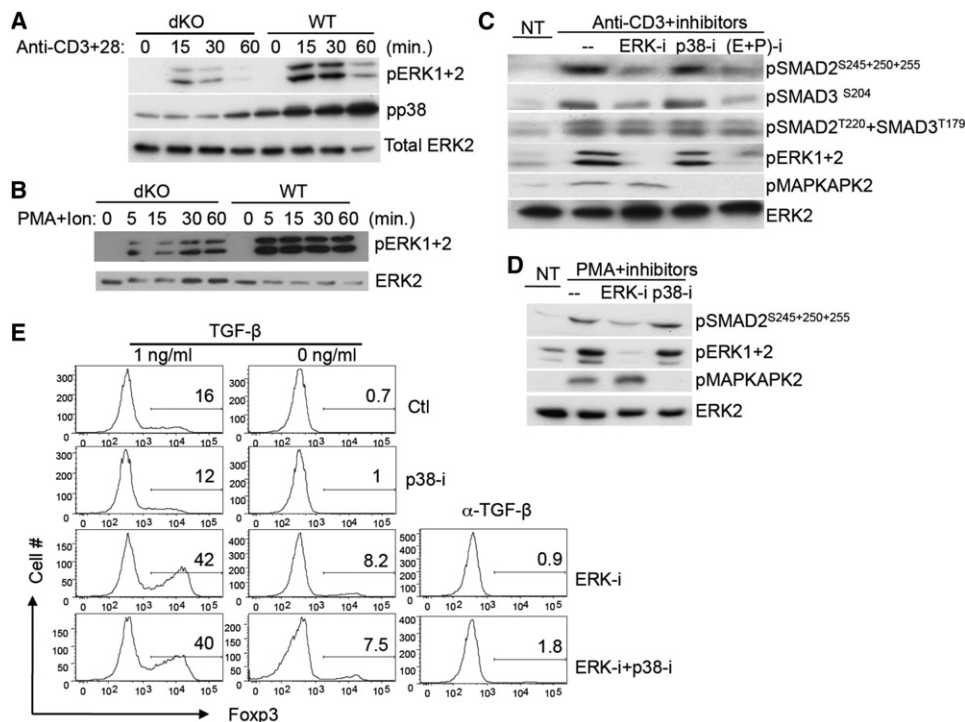


Figure 5. ERK1 and 2 Mediate the TCR-Induced SMAD Linker Region Phosphorylation and Inhibit the TGF- β Induced Treg Cell Differentiation

(A and B) WT or dKO CD4⁺ T cells were stimulated with CD3+CD28 antibodies (Anti-CD3+28) (A) or with PMA+ionomycin (PMA+Ion) (B) for the indicated time periods. Phosphorylation of ERK1 and 2 and p38 was determined by immunoblotting. Total ERK2 protein level was shown as a loading control.

(C and D) WT CD4⁺ T cells were activated with a CD3 antibody (C) or with PMA treatment (D) in the presence of vehicle DMSO (Ctl), or MEK1/2 inhibitor U0126 (ERK-i), or p38 inhibitor SB203580 (p38-i), or both ((E+P)-i) as indicated. Phosphorylations of SMAD2 or SMAD3 at their linker regions and phosphorylation of ERK1 and 2, and p38 at their activation loop, were determined by immunoblotting. ERK2 protein level was determined as a loading control. NT, nonstimulated cells.

(E) WT naive CD4 T cells were differentiated into Treg cells with either no TGF- β or 1 ng/ml TGF- β in the presence of no inhibitor (Ctl), or MEK1 inhibitor (ERK-i), or p38 inhibitor (p38-i), or both inhibitors (ERK-i+p38-i), as indicated. Foxp3⁺ Treg cells were determined 5 days later by flow cytometry as described in Figure 2. Data presented are representative of three independent experiments.

augmented the TGF- β -induced iTreg cell differentiation in vitro, similar to that found in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells. In contrast, inhibition of p38 activation had no effect on iTreg cell differentiation. In addition, inhibition of both p38 and ERK1 and 2 did not further increase the iTreg cell differentiation compared to ERK1 and 2 inhibition alone, further confirming that the TCR-induced SMAD2 and 3 phosphorylation at the linker region is mediated by ERK1 and 2, which negatively regulates TGF- β signaling during Th cell differentiation.

SMAD3 Linker Region Phosphorylation Mutant Induces More iTreg and Th17 Cells than WT SMAD3 in WT T Cells but Not in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T Cells

To directly test the role of the linker region phosphorylation of R-SMAD during Th cell differentiation, WT naive T cells were infected with retroviruses that expressed either GFP alone, or GFP plus WT SMAD3, or GFP plus EPSM-SMAD3, which is defective in its linker region phosphorylation (Calonge and Massagué, 1999). The infected T cells were then differentiated into either iTreg or Th17 cells in vitro. Expression of WT SMAD3 greatly enhanced both iTreg and Th17 cell differentiation as compared to the expression of the empty vector (Figure 6A). These results confirmed previous findings that SMAD3 is required for iTreg cell

differentiation and also suggested that SMAD3 was involved in Th17 cell differentiation. Importantly, we found that EPSM-SMAD3 had much higher activity than the WT SMAD3 in inducing either iTreg (93% versus 63%, respectively) or Th17 (41% versus 32%, respectively) cell differentiation (Figure 6A), indicating that phosphorylation of SMAD3 at the linker region is inhibitory to iTreg and Th17 cell differentiation.

If MEK2 and 3 suppress the iTreg and Th17 cell differentiation through the phosphorylation of R-SMAD at their linker regions, we would predict that WT SMAD3 may have comparable activity as the EPSM-SMAD3 in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells because the linker region of WT SMAD3 was hypophosphorylated in these cells (Figure 4D). Indeed, the WT SMAD3 induced similar Foxp3 expression as the EPSM-SMAD3 in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (Figure 6B, top). Consistent with R-SMAD having elevated activity in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells, WT SMAD3 also induced more Foxp3-expressing cells from the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (88%, Figure 6B) than from WT T cell (63%, Figure 6A) under the same condition. Moreover, EPSM-SMAD3 induced similar percentages of Foxp3-expressing cells from both the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells and WT T cells (Figures 6A and 6B, top). Finally, WT SMAD3 and EPSM-SMAD3 also

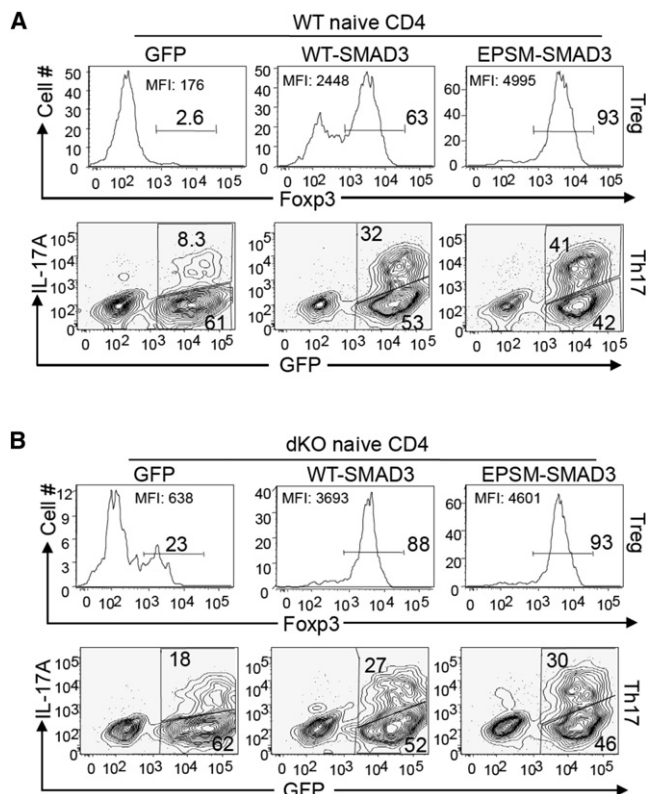


Figure 6. MEKK2 and MEKK3 Regulate Treg and Th17 Cell Differentiation through Mediating R-SMAD Linker Phosphorylation

WT (A) or dKO CD4⁺ T cells (B) were differentiated under the Treg or Th17 cell conditions, respectively, as described in Figure 2, except that the TGF- β (1 ng/ml) was added at the 24 hr time point when the cells were infected with empty retrovirus (GFP) or retroviruses that express WT-SMAD3 or EPSM-SMAD3. The infection was repeated once at 36 hr. Five days later, the infected cells were determined by GFP expression and analyzed for Foxp3⁺ cells directly (top) or analyzed for IL-17A⁺ cells after restimulation with PMA-ionomycin for 4 hr (bottom). The numbers in the graphs show the percentages of gated populations. Data are representative of two independent experiments.

induced comparable IL-17A-expressing cells in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (Figure 6B, bottom). Together with the results from the above biochemical study, our data strongly suggest that the TGF- β -dependent Th cell differentiation is negatively controlled by a TCR-MEKK2 and 3-ERK1 and 2 MAPK module, which phosphorylates SMAD2 and SMAD3 at their linker regions (Figure S5).

***Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} Mice Have Enhanced Th17 Cell Response**

To examine how MEKK2 and 3 deficiency in T cells may influence effector CD4 T cell differentiation and impact the development of acute inflammatory diseases, we used a well-characterized mouse autoimmune disease model, the experimental autoimmune encephalomyelitis (EAE) model. We immunized *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice with MOG₃₅₋₅₅ peptide and complete Freund's adjuvant (CFA) and monitored the development of EAE disease. Compared to WT mice, the

Map3k2^{-/-}*Map3k3*^{Lck-Cre/-} mice developed more severe disease after the immunization (Figure 7A) and recovered much slower from the paralysis. Similar results were obtained with the *Rag1*^{-/-} mice reconstituted with WT or *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} bone marrows (Figure 7B). Consistent with the more severe disease, the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice had more Th17 cells in both the central nervous system (CNS) and the spleen compared to the WT mice, whereas the Th1 cells appeared to be comparable (Figure 7C). Treg cells in the CNS were slightly reduced in ratio in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice, whereas their absolute numbers were comparable (Figure 7D and data not shown). As in the nonimmunized mice, the frequency of Foxp3⁺ Treg cells in the spleen of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice remained higher than that in the WT mice (Figure 7D).

To determine whether *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice produced more MOG-specific Th17 T cells, we tested the recall response of WT and *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells after in vivo MOG+CFA immunization. The antigen-induced production of IL-17A by *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells was significantly elevated over that by the WT T cells (Figure 7E), whereas IFN- γ production by *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells was also reduced (Figure 7F). These data together indicate that the T cell-specific deficiency of *Map3k2* and *Map3k3* results in augmented antigen-induced Th17 cell differentiation and consequent CNS accumulation, leading to more severe EAE disease.

DISCUSSION

TGF- β is a central regulator of Th cell differentiation but the underlying molecular mechanism by which the TGF- β signal is regulated during Th cell differentiation is not fully understood. Here we show that MEKK2 and MEKK3 deficiency in T cells resulted in hypersensitivity to the TGF- β signaling during iTreg and Th17 cell differentiation. We demonstrate that MEKK2 and MEKK3 are essential for the TCR-mediated activation of ERK1 and 2 and p38 MAPKs. Further, *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice develop more severe EAE disease and had more antigen-specific Th17 cells that preferentially accumulate in the central nervous system, suggesting that the MEKK2 and MEKK3 signaling pathway in T cells plays a crucial regulatory role in inflammation and autoimmunity. This study thus unravels an inhibitory mechanism that dampens the potential of TGF- β response in Th cell differentiation and reveals a critical interplay between the TCR-induced MAPK activation signal and the cytokine-induced differentiation signals during Th cell differentiation.

MEKK2 and MEKK3 are two highly homologous MAP3Ks. Although previous studies demonstrate that MEKK2 and MEKK3 have nonoverlapping function in vivo, our current study indicates that activation of either MEKK2 or MEKK3 alone is sufficient to suppress the TGF- β responses in T cells. In addition, either MEKK2 or MEKK3 is sufficient to activate ERK1 and ERK2 to phosphorylate SMAD2 or SMAD3 at their linker regions and to suppress R-SMAD-dependent transcriptional activity. These data indicate that MEKK2 and MEKK3 play overlapping roles in regulating the TGF- β response through ERK1 and 2 in Th cell differentiation.

Activation of ERK1 and 2 is mainly mediated by the Raf subgroup of MAP3Ks in T cells and other cell types. Other

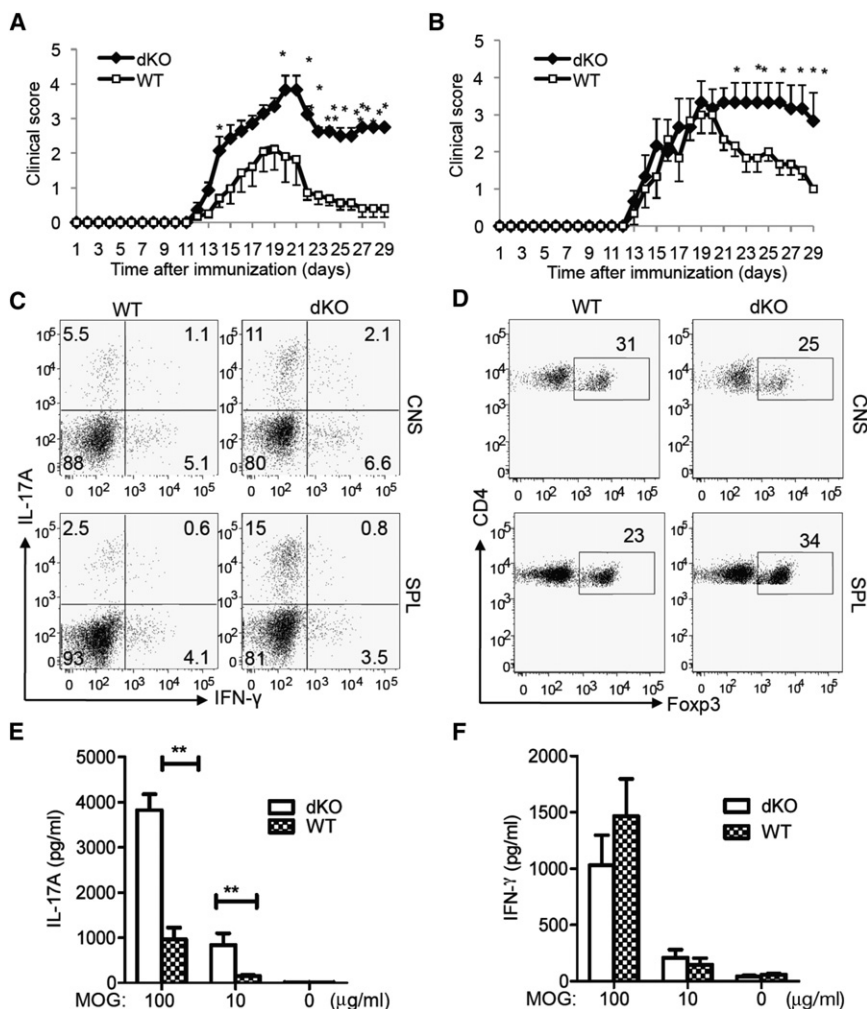


Figure 7. *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} Mice Exhibit More Severe EAE Disease and Accumulate More Th17 Cells in the Acute Inflammatory Response

(A) WT (n = 5) and dKO (n = 4) mice were immunized with MOG₃₅₋₅₅+CFA plus pertussis toxin to induce EAE disease. The immunized mice were observed for the indicated length of time and the mean clinical scores for the severity of EAE were determined.

(B) Sublethally irradiated *Rag1*^{-/-} mice were reconstituted with either dKO (n = 4) or WT (n = 5) bone marrow cells. Ten weeks after the reconstitution, EAE disease was induced and analyzed as described in (A).

Data shown are representative of two independent experiments. Error bars show standard error of the mean. **p < 0.01, *p < 0.05, by two-tailed Student's t test.

(C and D) EAE disease was induced in WT or dKO mice as described in (A). Twenty-eight days after the immunization, CD4⁺ T cells were gated from the total leukocytes in the central nervous system (CNS) or spleen (SPL) and further analyzed for frequencies of IL-17A⁺ and IFN- γ ⁺ cells (C) and Foxp3⁺ cells (D) by flow cytometry. The numbers in the plots indicate the percentages of the gated populations. Data shown are representative of three independent experiments.

(E and F) On day 8 after MOG+CFA immunization, WT or dKO splenocytes were restimulated with indicated concentrations of MOG peptide in vitro. The production of IL-17A (E) and IFN- γ (F) in culture supernatants was determined 3 days later by ELISA. Data shown are average values of triplicates. Error bars show standard deviation. The results are representative of four mice of each genotype from two independent experiments. **p < 0.01 by two-tailed Student's t test.

MAP3Ks such as MEKK1 (Yujiri et al., 1998), MEKK2 (Zhang et al., 2006), MEKK3 (Kim et al., 2007), and Tpl2 (Jager et al., 2010) are also implicated in ERK1 and 2 activation in T cells and other cell types either directly or indirectly. It is conceivable that these various alternative ERK1 and 2 activation pathways could allow cells to use the ubiquitous ERK1 and 2 MAPK module to selectively respond to distinct extracellular stimuli under different conditions. The specific MEKK2 and 3-ERK1 and 2 module in T cells may be one of such examples that is involved in transducing specifically the TCR signals to regulate the TGF- β pathway without affecting the Raf-ERK1 and 2-mediated growth signals during Th cell differentiation. Consistently, activation of ERK1 and 2 by cytokines such as IL-2 in T cells did not induce R-SMAD linker region phosphorylation. Therefore, our work suggests an interesting model that the TGF- β -dependent Th cell differentiation is negatively controlled by a TCR-MEKK2 and 3-ERK1 and 2 MAPK module, which phosphorylates SMAD2 and SMAD3 at their linker regions.

The NF- κ B pathway has been reported to play a positive role in Treg cell differentiation (Long et al., 2009; Ruan et al., 2009). We previously showed that MEKK3 plays a role in NF- κ B activation by the TLR ligands and proinflammatory cytokines

in fibroblasts (Huang et al., 2004; Yang et al., 2001). However, we did not find obvious augmentation of NF- κ B activation in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells, indicating that the augmented iTreg cell differentiation from naive *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells was probably not due to the altered NF- κ B pathway. Although studies from another group reported that MEKK3 was required for NF- κ B activation in T cells (Shinohara et al., 2009), we found no obvious defect in the TCR-induced IKK β activation and I κ B α degradation in either *Map3k3*^{Lck-Cre/-} or *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells (data not shown). Interestingly, the induction of p65 phosphorylation at Ser534 after TCR stimulation was impaired in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells, supporting a role of MEKK2 and MEKK3 in NF- κ B pathway activation in T cells (data not shown). Nevertheless, we believe that this defect is most probably unrelated to the increased Treg cell differentiation of *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} T cells because defective NF- κ B activation should inhibit but not augment the Treg cell differentiation (Long et al., 2009; Ruan et al., 2009).

The peripheral T cells in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice appeared to be overtly activated, but the cause of this change is unclear. However, this is probably not the reason why the

Map3k2^{-/-}*Map3k3*^{Lck-Cre/-} T cells are hypersensitive to TGF- β signal because naive peripheral T cells or thymic CD4 SP T cells that have not been previously activated were also hypersensitive to TGF- β signal. Interestingly, although the frequencies of iTreg cells were increased in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice and they were able to suppress naive T cell proliferation in vitro, they failed to suppress the activated T cell phenotype in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice. Similarly, the Treg cells in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice failed to suppress the pathogenesis of EAE. In this regard, expansion of iTreg cells has been frequently observed in autoinflammatory conditions such as in the CNS of a mouse EAE model (Korn et al., 2007) or in the inflamed joints of rheumatoid arthritis patients (Ruprecht et al., 2005). One possible cause of this phenomenon could be the overproduction of IL-6 induced by IL-17A in the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice, which has been shown to dampen the iTreg cell-mediated suppression of naive T cell activation (Pasare and Medzhitov, 2003).

Finally, the augmented T cell activation and the increased Th17 cells in the periphery may predispose the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice to autoimmune diseases. However, no spontaneous autoimmune syndromes have been observed in *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice up to 15 months old. In contrast, under acute inflammatory condition, after immunization with MOG+CFA, the *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice suffered more severe disease than the WT mice, which is most probably caused by the enhanced production of MOG-specific Th17 cells and their CNS accumulation. It is therefore possible that MEKK2 and MEKK3 may positively affect immune tolerance dependent on the inflammatory status, similar to the role of TGF- β in autoimmune diseases. Interestingly, similar to our observation, mice with ERK1 deficiency also had more severe EAE disease after the MOG/CFA immunization (Agrawal et al., 2006). Future studies of the roles of MEKK2 and MEKK3 in immune tolerance and autoimmunity will be rewarding.

EXPERIMENTAL PROCEDURES

Mice

Mekk3 germline KO mice, *Map3k3*^{fl/fl} mice, *MAP3k3* T cell conditional-deleted mice, and *Map3k2*^{-/-} mice were described before and were bred with C57BL/6 mice for more than 10 generations (Cheng et al., 2000; Guo et al., 2002; Wang et al., 2009). Lck-Cre transgenic mice in C57BL/6 background, C57BL/6 *Rag1*^{-/-}, WT C57BL/6 mice, and C57BL/6 CD45.1 (B6.SJL) congenic mice were obtained from the Jackson Laboratory. All mice were maintained under pathogen-free conditions. Unless indicated, all mice were used between 10 and 15 weeks old. All animal experimentation was conducted in accordance with institutional guidelines.

Bone Marrow Transplantation

Bone marrow cells were isolated from *Map3k2*^{-/-}*Map3k3*^{Lck-Cre/-} mice, WT mice, or B6.SJL mice. Five million cells were injected intravenously into *Rag1*^{-/-} mice, which received a sublethal irradiation (400 rad) 1 day before. Chimeric mice were analyzed 6 to 8 weeks after transplantation.

Chemical Inhibitors

The following inhibitors were used: MEK inhibitor (PD98059, 5 μ M), MEK1/2 inhibitor (U0126, 5 μ M), p38 inhibitor (SB20190, 5 μ M), CDK inhibitor (flavopiridol, 0.3 μ M), GSK3 α/β inhibitor (SB-216763, 5 μ M), and TGF- β R inhibitor (SB431542). All inhibitors were obtained from Sigma.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/gds>) under the accession number GSE26987.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2011.01.017.

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